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13. ABSTRACT *(Maximum 200 Words)*

The aim of this proposal is to purify a sufficient amount of VEGI, a novel inhibitor of angiogenesis we discovered recently, in order to determine its anticancer activity in animal models. Initially we planned to express the protein in insect cells; however, we found out that we can express the protein in E. coli with good yield and very high activity. We have developed two bench-top procedures to purify VEGI protein. When expressed in E. coli, VEGI protein is basically found in the inclusion bodies. Upon refolding, the protein, which is tagged with 10-histidine residues at the N-terminus, is purified by a one-step affinity chromatography using Ni-sepharose resin. The second purification procedure involves dissolving the inclusion bodies in SDS and purify the denatured VEGI protein by using a gel filtration column; the purified VEGI protein is then refolded by dilution (Scheme 2). The yield from either procedure is 2-5 mg/L, which is reasonable for bench-top production. The specific activity of the protein preparation, as determined by the IC50 value for the inhibition of endothelial cell growth, is 6 ng/ml, representing nearly 500-fold improvement as compared to our earlier preparations. These procedures are simple and ready for scale-up studies.

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Lynnan L. 8/24/99

PI - Signature Date

Table of Content

INTRODUCTION

We have recently identified a new protein, named vascular endothelial cell growth inhibitor (VEGI), that is expressed only by endothelial cells and can inhibit the growth of endothelial cells but not that of other types of cells examined. The IC_{50} value for the inhibition is 6 ng/ml (0.3 nM). The protein also inhibits the formation of capillary-like structures by endothelial cells in collagen gels, and the growth of capillaries into collagen gels placed on the chick chorioallantoic membrane. Chinese hamster ovary cells overexpressing a secreted form of the protein inhibits the growth of breast cancer and prostate cancer xenograft tumors in nude mice when subcutaneously coinjected with the cancer cells. Transfection of mouse colon cancer cells with the secreted VEGI led to nearly complete inhibition of the ability of the cancer cells to grow tumors in C57BL black mice. The gene is expressed in many normal tissues, but is down-regulated in proliferating endothelial cells. We hypothesize that VEGI functions as a negative regulator of angiogenesis to maintain the quiescence of the normal vasculature, or terminate an angiogenesis process. We proposed to purify a sufficient amount of active VEGI protein in order to determine its anticancer activity in animal models.

BODY

Improved Specific Activity of the VEGI Protein: We have constructed a fusion protein in which residues 29-174 of the VEGI protein is linked to a poly-histidine tag. The poly-His tag is used to facilitate protein purification and detection. The protein was expressed in E. coli, purified to homogeneity, and examined in a variety of cellular assays. The recombinant protein was found to potently inhibit the proliferation of adult bovine aortic endothelial (ABAE) cells (Fig. 1). Nearly complete inhibition of the growth of the ABAE cells was achieved at 100 ng/ml of the recombinant protein. The half-maximum inhibitory concentration (IC_{50}) was about 6 ng/ml (about 2 x 10^{-10} M). Similar inhibition of HUVE cells, with an IC_{50} of 60 ng/ml, was also observed. In contrast, the protein had no effect on the growth of human vascular smooth muscle cells, human breast cancer cells (MDA-MB-231), or NIH 3T3 cells under similar experimental conditions. The protein also had no effect on the proliferation of human T-cells and bone marrow stromal cells (data not shown). These results suggest that the new protein is not only specifically expressed by endothelial cells, but also targets highly specifically on endothelial cells, at least with regard to the regulation of proliferation. These data have now been published in two manuscripts (Zhai et al, 1999; Zhai et al, 1999; see Appendices).

As I mentioned in the grant application, the amino acid sequence of VEGI suggests the presence of a transmembrane domain dear the N-terminus. In order to express VEGI as a soluble protein, it is necessary to delete this highly hydrophobic domain. The improvement of the protein activity is most like due to a better selection of the truncation site. We have tried a variety of truncation sites. Previously residues 34-174 were used in earlier construct. The current construct (residues 29-174) gives us the best activity, which is improved by nearly 500-fold as compared to the protein preparation we had when the grant application was first written.

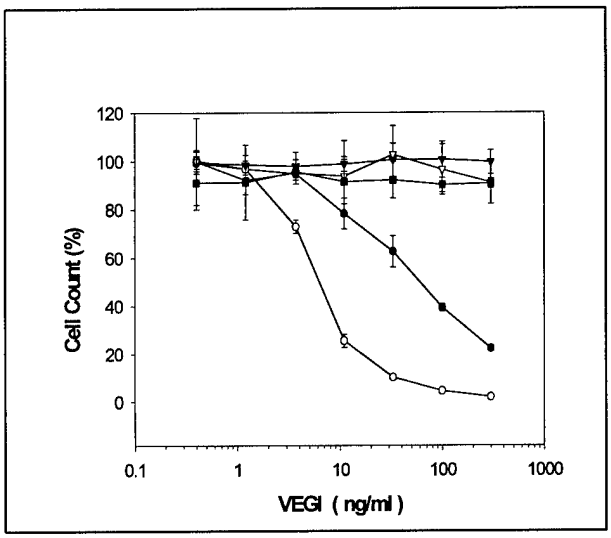


FIG. 1: Specific inhibition of endothelial cell proliferation by VEGI. Closed circles: human umbilical cord vein endothelial cells (HUVE); Open circles: adult bovine aortic endothelial cells (ABAE); Closed triangles: human vascular smooth muscle cells (HVSM); Open triangles: human breast cancer cells (MDA-MB-231); Closed squares: NIH3T3 mouse fibroblast cells. Cells were seeded in triplicate at 8000 cells/well in 24-well plates. Culture conditions are described under Methods. The media were changed once on day 3. The number of viable cells was determined on day 6 by using a Coulter counter. Mean values and standard deviations are presented as a function of VEGI concentrations.

Purification Schemes: Initially I proposed to make an Fc-fusion protein. The proposal is modified because the Fc-fragment tends to form dimers, while we suspect that our protein may need to form a trimer to be active, similar to other members of the TNF superfamily. In addition, we initially planned to express VEGI in insect cells, for fear that the low activity of earlier VEGI preparation may result from lack of post-translational modifications. However, since the E. coli-expressed new construct exhibited potent activity, we have decided to use the bacterial system because it is simple and effective. Two purification schemes are enclosed.

Scheme 1: Purification of VEGI by Ni-Agarose Chromatography

Expression:

1. Pick a single colony to grow in 2.5 ml of LB media containing ampicillin (100μg/ml), 37°C, 8-9hrs.
2. Transfer 2ml of the cultures to 100 ml of LB. Grow the cultures at 37°C overnight.
3. Transfer 20 ml of the overnight cultures into 1 L of LB media containing ampicillin (100μg /ml) in a 2 L of flask. Incubate at 37°C with vigorous shaking(300rpm), until the OD_{600} is 0.6-0.9 (approximately 2-3hrs)
4. Induce expression by adding IPTG to final concentration of 1mM.
5. Grow the cultures for an additional 3 hours.
6. Harvest the cells by centrifugation at 8000rpm for 10min, and wash the cells twice with PBS.
7. Store the cells at –70°C.

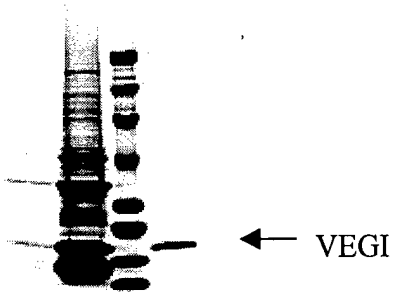
Purification:

1. Freeze and thaw the cells 2-3 times.
2. Sonicate briefly to resuspend the pellet thoroughly and to shear the DNA.
3. Wash the pellet 6 times with wash buffer.

Wash Buffer:

HEPES-KOH	25mM (pH7.6)
EDTA	10mM
NaCl	100mM
Triton X-100	1%
PMSF	0.5mM

8. Dissolve the inclusion body with 10ml of 50% acetic acid and incubate at room temp. for 2-3 hrs.
9. Centrifuge at 13,000 rpm for 15 minutes to remove undissolved pellets.
10. Refold the protein by diluting the solution 80X with 20mM of HEPES-KOH, pH 7.6; place the diluted solution at RT for 48 hrs.
11. Purify the VEGI by Ni-Agarose chromatography.
12. Store the samples at –70°C.



1 2 3
1: inclusion body
2: MW standard
3: purified VEGI

Scheme 2: Purification of VEGI by Gelfiltration Chromatography

1 L fermentation of VEGI gene transformed *E coli*

↓ 8000 rpm*10 min
Wash with PBS twice

Pellet

↓ Sonication
13000 rpm**20 min

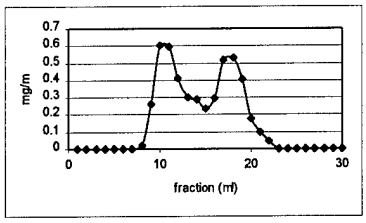
Pellet (inclusion body)

↓ Washing with pH 8.0, 0.05 mM Tris-HCl buffer containing 1% Triton X-100
13000 rpm*20 min

Precipitate (repeat washing 6 times)

↓ Take ¼ precipitate
Dissolved in 1-2 ml denature buffer
13000 rpm*20

Supernatant

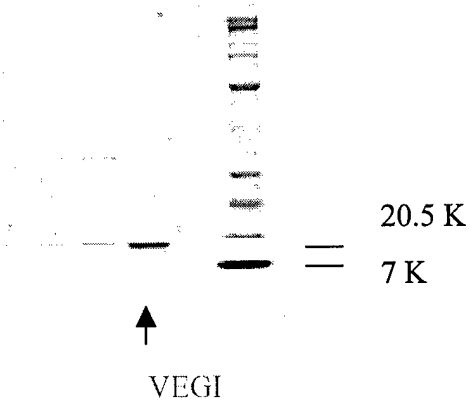


↓ Superose-12 gel filtration

The second peak

↓ 1:10-100 diluted with refolding buffer
48 h

Refolded VEGI (1-2 mg)



KEY RESEARCH ACCOMPLISHMENT

- Constructed a VEGI fusion protein with significantly improved activity
- Established purification schemes that are simple and ready to scale-up

REPORTABLE OUTCOMES

Manuscripts:

Zhai et al. VEGI: a novel cytokine of the TNF family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas in vivo. The FASEB Journal 13:181-189, 1999

Zhai et al. Inhibition of Angiogenesis and Breast Cancer Xenograft Tumor Growth by VEGI, a Novel Cytokine of the TNF Superfamily. Int. J. Cancer 82:131-136, 1999

Meeting Abstracts:

Yu, JY, Hayes, AJ, and Li, LY: VEGI, a novel cytokine of the TNF superfamily, arrests endothelial cell growth at G0/G1 phase. AACR Annual Meeting, Philadelphia, PA, 1999

Zhao, QH, Yu, JY, and Li, LY: VEGI, an Endothelial-Specific Negative Regulator of Angiogenesis, Is Highly Resistant to Heat and Forms a Trimer in Solution. ASBMB Annual Conference, San Francisco, CA, 1999

Yu, JY, Zhao QH, Hayes, AJ, Lippman, ME, and Li, LY: Anticancer Therapeutic Potential of VEGI. NCI SPORE Investigators Workshop, Rockville, MD, 1999

Grants Generated:

Agency: NIH/NHLBI. ID: 1 R01 HL60660-01
Title: A Novel Negative Regulator of Angiogenesis
Start Date 7/1/98 End Date 4/30/02
Role: PI. Percentage of time: 40%
Total Direct: $814,980, FY Amount: $204,245

Agency: NIH/NCI Rapid Access to Intervention Development Program
Title: Anticancer potential of VEGI
Role: PI. Percentage of time: 10%
Start Date 7/1/99 End Date 6/30/02
Total Direct: The RAID Program will make the NCI Frederick Drug Development Facility available to VEGI project in order to carry out pre-clinical trials; Estimated direct cost $2,000,000

CONCLUSIONS

We have accomplished the first half of the tasks according to schedule. The activity of the VEGI protein preparation is improved by nearly 500-fold as compared to the preparation we had when the grant application was written. We are now in the process of purifying a sufficient amount of the VEGI protein in order to examine its anti-cancer activity in breast cancer mouse models.

APENDICES

Zhai et al. VEGI: a novel cytokine of the TNF family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas in vivo. The FASEB Journal 13:181-189, 1999

Zhai et al. Inhibition of Angiogenesis and Breast Cancer Xenograft Tumor Growth by VEGI, a Novel Cytokine of the TNF Superfamily. Int. J. Cancer 82:131-136, 1999

VEGI, a novel cytokine of the tumor necrosis factor family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas *in vivo*

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ABSTRACT **A novel member of the tumor necrosis factor (TNF) family has been identified from the human umbilical vein endothelial cell cDNA library, named vascular endothelial growth inhibitor (VEGI). The VEGI gene was mapped to human chromosome 9q32. The cDNA for VEGI encodes a protein of 174 amino acid residues with the characteristics of a type II transmembrane protein. Its amino acid sequence is 20–30% identical to other members of the TNF family. Unlike other members of the TNF family, VEGI is expressed predominantly in endothelial cells. Local production of a secreted form of VEGI via gene transfer caused complete suppression of the growth of MC-38 murine colon cancers in syngeneic C57BL/6 mice. Histological examination showed marked reduction of vascularization in MC-38 tumors that expressed soluble but not membrane-bound VEGI or were transfected with control vector. The conditioned media from soluble VEGI-expressing cells showed marked inhibitory effect on *in vitro* proliferation of adult bovine aortic endothelial cells. Our data suggest that VEGI is a novel angiogenesis inhibitor of the TNF family and functions in part by directly inhibiting endothelial cell proliferation. The results further suggest that VEGI maybe highly valuable toward angiogenesis-based cancer therapy.—Zhai, Y., Ni, J., Jiang, G.-W., Lu, J., Xing, L., Lincoln, C., Carter, K. C., Janat, F., Kozak, D., Xu, S., Rojas, L., Aggarwal, B. B., Ruben, S., Li, L.-Y., Gentz, R., Yu, G.-L. VEGI, a novel cytokine of the tumor necrosis factor family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas *in vivo*. *FASEB J.* 13, 181–189 (1999)**

Key Words: TNF · cytokine · anti-tumor · anti-angiogenesis · EST

TUMOR NECROSIS FACTOR α (TNFα)[2] was first identified as a cytokine that caused the abrogation of tumors and has been investigated for clinical uses toward the treatment of various tumors (1–5). TNFα is also implicated in pathological conditions such as septic shock, autoimmune disorders, and graft-versus-host disease. However, TNFα is a highly pleiotropic cytokine whose biological activities include cell cytotoxicity, mediation of cell proliferation, and immune responses, depending on the target cells. These diverse functions result in general toxicity *in vivo* (1–5). The TNF ligand and receptor families are emerging gene families that play important roles in immune regulation and inflammation. In addition to TNFα, lymphotoxin (LTα or TNFβ), lymphotoxin β (LTβ), and ligands for CD27, CD30, CD40, OX40, 4-1BB, and Fas, which were cloned by classical biochemical approaches and receptor–ligand interactions, new members of the TNF ligand family, TRAIL (APO-2L) (6, 7), TWEAK (8), TRANCE/RANK (9, 10), and LIGHT (11), have been identified from an expressed sequence tag (EST) database. In this report, we describe the identification of a new member of the TNF ligand family. Unlike other members of this family, vascular endothelial growth inhibitor (VEGI) is produced predominantly by the endothelial cells. We have shown that VEGI is a vascular

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[2] Abbreviations: ABAE, adult bovine aortic endothelial cells; bFGF, basic fibroblast growth factor; EST, expression sequence tag; HUVEC, human umbilical vein endothelial cells; HMMVEC, human myometrialuterine microvascular endothelial cells; HPAEC, human pulmonary artery endothelial cells; HIAEC, human iliac artery endothelial cells; HCAEC, human coronary artery endothelial cells; IL, interleukin; LT, lymphotoxin; mAb, monoclonal antibody; PCR, polymerase chain reaction; TNF, tumor necrosis factor; VE, venous endothelial; VEGI, vascular endothelial growth inhibitor; vWF, von Willebrand factor.

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endothelial growth inhibitor that suppresses the growth of colon carcinoma in mice via gene transfer.

MATERIALS AND METHODS

Cells and reagents

The murine colon adenocarcinoma cells, MC-38, were kindly provided by Dr. Steven A. Rosenberg (National Cancer Institute, Bethesda, Md.). The tumor cells were grown and maintained in RPMI medium containing 10% FCS. Normal human endothelial cells were purchased from Clonetics Corp. (San Diego, Calif.); these primary endothelial cells were originally derived from different organs including human umbilical vein endothelial cells (HUVEC), human myometrialuterine microvascular endothelial cells (HMMVEC), human pulmonary artery endothelial cells (HPAEC), human iliac artery endothelial cells (HIAEC), and human coronary artery endothelial cells (HCAEC). The cells were cultured according to manufacture's procedure. Restriction enzymes, random primer DNA labeling kit were obtained from Boehringer Mannheim (Indianapolis, Ind.). [^{32}P] dCTP was purchased from Amersham Corp. (Arlington Heights, Ill.).

Cloning and sequencing of VEGI full-length cDNA

The cDNA clone containing the original EST was used to synthesize ^{32}P-labeled probes. A lambda zap (Stratagene, San Diego, Calif.) cDNA library constructed using HUVEC cells was screened by conventional methods using nylon filter lifts. Six positive clones were isolated from approximately 10^6 plaques. The cDNA clones were converted into pBlueScript (SK$^+$) plasmid (Stratagene) using helper phages by following a protocol provided by the manufacturer. The insert sizes of the cDNA clones were determined by polymerase chain reaction (PCR) using a vector-specific oligonucleotide (M13 reverse primer) and a gene-specific oligonucleotide. A cDNA clone containing the largest insert (4.5 kb) was sequenced using the Applied Biosystems automated sequencer, ABI 373 (Perkin-Elmer, Norwalk, Conn.). The partial sequence of the same gene was recently reported in which no function was identified (13).

Northern blot analyses

VEGI mRNA expression was detected on multiple tissue blots purchased from Clontech Laboratories, Inc. (Palo Alto, Calif.). Hybridization of the blots with radiolabeled VEGI probe was performed using manufacturer's suggested conditions. For RNA blots containing the RNA from cell lines, total RNA was purified using RNAzol (Qiagen, Chatsworth, Calif.) and electrophoresed on 1.2% formaldehyde agarose gel and blotted on a Nylon filter. Northern blot analysis was performed using standard procedure.

Transfection of VEGI into MC-38 cells

A fusion protein (VEGI/IL-6) consisting of the secretion signal of interleukin-6 (IL-6) and residues 23–174 of VEGI was constructed by PCR (5′-primer: 5′-GCG GGATCCG CCACCATGAA CTCCTTCTCC ACAAGCGCCT TCGGTCCAGT TGCCTTCTCC CTGGGGCTGC TCCTGGTGTT GCCTGCTGCC TTCCCTGCCC CAGTTGTGAG AC-3′, containing a BamH I restriction endonuclease site, the first 84 bases of IL-6 coding sequence and 18 bases of VEGI starting from Pro23; 3-primer: 5′-CGC GGATCCG ATATTTGCTC TCCTCCTCA-3′, containing a BamH I restriction endonuclease site and a stop codon). For transfection of the full-length VEGI and IL-6/VEGI into MC-38 cells, the constructs were generated by cloning the inserts into the pcDNA3 expression vector (Invitrogen, Carlsbad, Calif.). Subsequent to transfection, G418 selection, and cloning, three clones for each constructs were picked for tumorigenicity studies. The expression of VEGI in MC-38 cells was confirmed by Northern blot analysis.

In vivo tumorigenicity assay

Female C57BL/6 mice, 6–7 wk old, were purchased from Harlan Sprague Dawley (Indianapolis, Ind.). Various groups of MC-38 cells (2×10^5 cells/mouse) were injected into C57BL/6 mice. Mice were then ear tagged and randomized and tumors were measured twice weekly in a blinded fashion. The tumor size was assessed by measuring perpendicular diameters with a caliper and calculated by multiplying the measurements of diameters in two dimensions. Data are represented as the mean ± SD of nine mice in each group and the experiments were repeated with similar results.

Immunohistochemical analyses

Excised tumors were fixed in formalin and five-micron sections were cut and mounted on Superfrost Plus slides. Sections were stained with the following antibodies according to the manufacture's procedure: hematoxylin and eosin (H&E) or GR-1 (Ly-6G), a rat anti-mouse monoclonal antibody (mAb) that specifically recognizes neutrophils/granulocytes (PharMingen, San Diego, Calif.), or rabbit anti-human von Willebrand factor (vWF) mAb, which specifically recognizes the endothelial cells (DAKO Corp., Carpinteria, Calif.), or MMAC, a rabbit anti-mouse mAb that specifically recognizes monocytes and macrophages. The amounts of Gr-1+ neutrophils, vWF+ endothelial cells, or MMAC+ macrophages and the total tumor areas of each tumor were determined by the use of BioQuant-True image analysis system (R&M Biometrics, Nashville, Tenn.).

Cell proliferation assay

MC-38 tumor cells or the adult bovine aortic endothelial cells (ABAE) were harvested and seeded in wells of a 96-well plate at 3000–5000 cells/well in the appropriate growth medium containing 10% fetal bovine serum and 1 ng/ml of basic fibroblast growth factor (bFGF). The conditioned media collected from the soluble VEGI-expressing or the neo vector-transfected cells were then added at a final dilution 1:10 into each well. The cells were incubated in a final volume of 200 μl for a 4–5 days. Alamar blue was added to each well to a final concentration of 10%. The cells were incubated for 4 h. Cell viability was measured by reading in a CytoFluor fluorescence reader with excitation at 530 nm and emission at 590 nm. All assays were done in triplicates.

RESULTS

VEGI is a new member of the TNF superfamily

To identify an autocrine inhibitor of angiogenesis specific to endothelial cells, we have constructed eight cDNA libraries using RNA derived from various endothelial cells, and generated approximately 3 ×

A

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1       TTCTCTCCTGTCTCTCTCTTTCATTCATACACTGAGTCATTCAGAGATGGCTTCTCTCCA
60      ACTCGGAGCTGCAAGTAATTCTGGATCTGGTCACACACACAAAGTCCCCAGAGTTGCCAA
121     TTTATCTAGTTCATCTGTGCCTGTTCAAGATGATGTAACTAAACATTTACCTTCAGGGAG
181     GTGTTTCCAAAGAATTTTCATCGATATATAGAAATCAAGAGAAAATCCATACTATCACCA
241     AATCAAGAGAAATTCCATACTATCACCAGTTGGCCAACTTTCCAAGTCTAGTGCAGAAAT
301     CCAAGGCACCTCACACCTAGAGTTCCTATACCTCTGAGACTCCAGAGGAAAGAACAAGAC
361     AGTGCAGAAGGATATGTTAGAACCCACTGAAAACCTAGAAGGTTAAAAAGGAAGCATACC
421     CTCCTGACCTATAAGAAAATTTTCAGTCTGCAGGGGGATATCCTTGTGGCCCAAGACATT
481     GGTGTTATCATTTGACTAAGAGGAAATTATTTGTGGTGAGCTCTGAGTGAGGATTAGGAC
541     CAGGGAGATGCCAAGTTTCTATCACTTACCTCATGCCTGTAAGACAAGTGTTTTGTTCCA
601     ATTGATGAATGGGGATAAAACAGTTCAGCCAATCACTTATGGGGCAAAGAATGGGAATTT
661     GAAGGGTCTGGTGCCTGGCCTTGTCATACGTAAACAAGAGAGGCATCGATGAGTTTTATC
721     TGAGTCATTTGGGAAAGGATAATTCTTGCAGCAAGCCATTTTCCTAAACACAGAAGAATA
781     GGGGGATTCCTTAACCTTCATTGTTCTCCAGGATCATAGGTCTCAGGTAAAATTAAAAAT
841     TTTCAGGTCAGACCACTCAGTCTCAGAAAGGCAAAGTAATTTGCCCCAGGTCACTAGTCC
901     AAGATGTTATTCTCTTTGAACAAATGTGTATGTCCAGTCACATATTCTTCATTCATTCCT
961     CCCCAAAGCAGTTTTTAGCTGTTAGGTATATTCGATCACTTTAGTCTATTTTGAAAATGA
1021    TATGAGACGCTTTTTTAAGCAAAGTCTACAGTTTCCCAATGAGAAAATTAATCCTCTTTCT
        M  R  R  F  L  S  K  V  Y  S  F  P  M  R  K  L  I  L  F  L

1081    TGTCTTTCCAGTTGTGAGACAAACTCCCACACAGCACTTTAAAAATCAGTTCCCAGCTCT
        V  F  P  V  V  R  Q  T  P  T  Q  H  F  K  N  Q  F  P  A  L

1141    GCACTGGGAACATGAACTAGGCCTGGCCTTCACCAAGAACCGAATGAACTATACCAACAA
        H  W  E  H  E  L  G  L  A  F  T  K  N  R  M  N  Y  T  N  K

1201    ATTCCTGCTGATCCCAGAGTCGGGAGACTACTTCATTTACTCCCAGGTCACATTCCGTGG
        F  L  L  I  P  E  S  G  D  Y  F  I  Y  S  Q  V  T  F  R  G

1261    GATGACCTCTGAGTGCAGTGAAATCAGACAAGCAGGCCGACCAAACAAGCCAGACTCCAT
        M  T  S  E  C  S  E  I  R  Q  A  G  R  P  N  K  P  D  S  I

1321    CACTGTGGTCATCACCAAGGTAACAGACAGCTACCCTGAGCCAACCCAGCTCCTCATGGG
        T  V  V  I  T  K  V  T  D  S  Y  P  E  P  T  Q  L  L  M  G

1381    GACCAAGTCTGTATGCGAAGTAGGTAGCAACTGGTTCCAGCCCATCTACCTCGGAGCCAT
        T  K  S  V  C  E  V  G  S  N  W  F  Q  P  I  Y  L  G  A  M

1441    GTTCTCCTTGCAAGAAGGGGACAAGCTAATGGTGAACGTCAGTGACATCTCTTTGGTGGA
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1501    TTACACAAAAGAAGATAAAACCTTCTTTGGAGCCTTCTTACTATAGGAGGAGAGCAAATA
        Y  T  K  E  D  K  T  F  F  G  A  F  L  L

1561    TCATTATATGAAAGTCCTCTGCCACCGAGTTCCTAATTTTCTTTGTTCAAATGTAATTAT
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1681    ATGAAATTTGGGGCCAAAATTTCACACTTCATGTGCCTTACTGATGAGAGTACTAACTGG
1741    AAAAAGGCTGAAGAGAGCAAATATATTATTAAGATGGGTTGGAGGATTGGCGAGTTTCTA
1801    AATATTAAGACACTGATCACTAAATGAATGGATGATCTACTCGGGTCAGGATTGAAAGAG
1861    AAATATTTCAACACCTCCTGCTATACAATGGTCACCAGTGGTCCAGTTATTGTTCAATTT
1921    GATCATAAATTTGCTTCAATTCAGGAGCTTTGAAGGAAGTCCAAGGAAAGCTCTAGAAAA
1981    CAGTATAAACTTTCAGAGGCAAAATCCTTCACCAATTTTTCCACATACTTTCATGCCTTG
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2161    ATCTAAATAAGGAAGTATGAGTTTCACATGTATATCAAAAATACAACAGTTGCTTGTATT
2221    CAGTAGAGTTTTCTTGCCCACCTATTTTGTGCTGGGTTCTACCTTAACCCAGAAGACACT
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2341    CAAACATCAGTCAACATACTCTAAAGAATAACTCCAAGTCTTGGCCAGGCGCAGTGGCTC
2401    ACACCTGTAATCCCAACACTTTGGGAGGCCAAGGTGGGTGGATCATCTAAGGCCGGGAGT
2461    TCAAGACCAGCCTGACCAACGTGGAGAAACCCCATCTCTACTAAAAATACAAAATTAGCC
2521    GGGCGTGGTAGCGCATGGCTGTAATCCTGGCTACTCAGGAGGCCGAGGCAGAAGAATTGC
2581    TTGAACTGGGGAGGCAGAGGTTGCGGTGAGCCCAGATCGCGCCATTGCACTCCAGCCTGG
2641    GTAACAAGAGCAAAACTCTGTCCAAAAAAAAAAAAAAAAAAAAA
```

Figure 1. *A*) VEGI cDNA and deduced amino acid sequence. Numbers refer to the positions of nucleotides and amino acids. The putative transmembrane domain of VEGI is underlined. *B*) Alignment of the predicted amino acid sequence of VEGI, TNFα, TNFβ, and the Fas ligand (FasL). The extracellular regions of the proteins were compared. Numbers on the right indicate the position of the last amino acid of each line. Shaded amino acids are amino acids conserved among at least two members. An overall homology of 20–30% is found. Note that two tryptophan (W42 and W132) and four tyrosine residues (Y70, Y73, Y111, and Y136) are conserved in all four sequences. Residues N59 and N152 may be potential glycosylation sites.

10^4 ESTs (12). The ESTs were compared with about 1 million ESTs in the human genome databases derived from a wide variety of types of cells. ESTs unique to endothelial cells were further characterized for sequence homology to known protein families, particularly cytokines. The deduced amino acid sequences of one group of ESTs contained consensus amino acid sequence characteristics of the TNF family. A 4.5 kb cDNA clone containing the full-length gene was obtained based on these ESTs by screening an HUVEC library. An approximately 2.6 kb sequence of the clone is shown in **Fig. 1***A*. The cDNA

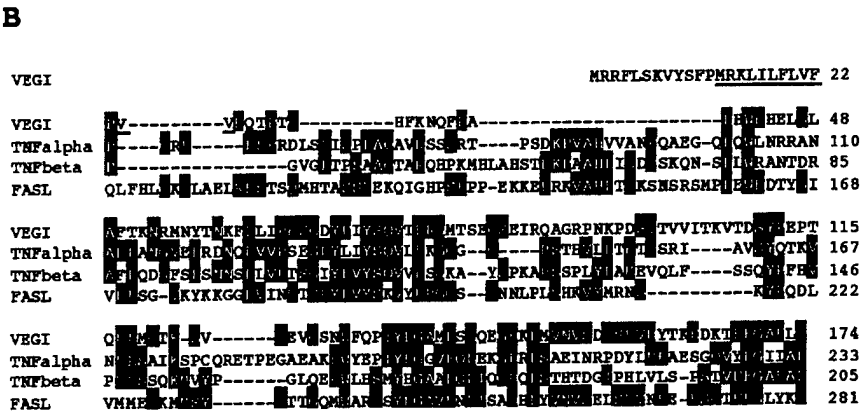

Figure 1. *(continued)*

encodes an open reading frame of 174 amino acids. There is an in-frame stop codon upstream of the predicted initiation codon, indicating that the translation cannot start further upstream. It is interesting that there are long untranslated regions at both ends of open reading frame. It is not clear whether these long untranslated regions play any functional role. The predicted protein VEGI exhibits 20–30% sequence homology to human TNFα, TNFα, and the Fas ligand (Fig. 1*B*), similar to that among other TNF family members. In addition, two tryptophan (W42 and W132) and four tyrosine residues (Y70, Y73, Y111, and Y136) are conserved in all four sequences. Residues N59 and N152 may be potential glycosylation sites. A protein with a molecular mass of 22 kDa was produced in an *in vitro* transcription and translation experiment using the cDNA clone as a template (data not shown), consistent with the predicted open reading frame. Hydrophobicity analysis of the protein predicts a 13 amino acid hydrophobic region that follows the amino-terminal segment of 12 residues. This is characteristic of type II transmembrane proteins, with the carboxyl terminus on the exterior cell surface (residues 26–174), a single transmembrane domain, and a short cytoplasmic tail. Thus, this novel protein resembles other TNF family members, with the exception of TNFβ (1). Since the new protein was subsequently found to be able to inhibit endothelial cell growth (see below), it is designated VEGI. The accession number for human VEGI cDNA in GenBank is AF039390.

VEGI is specifically expressed in endothelial cells

The fluorescence in situ hybridization mapping procedure was used to investigate VEGI localization. Detailed analyses of seven individual chromosomes indicated that the VEGI gene is located within band 9q32. Using multiple tissue Northern blots, the VEGI transcript was found to be expressed in placenta, lung, kidney, skeletal muscle, pancreas, spleen, prostate, small intestine, and colon. Little VEGI signal was detected in heart, brain, liver, thymus, testis, ovary, and peripheral blood lymphocytes (**Fig. 2*A***). The size of VEGI mRNA is approximately 6.5 kb. It is unusual for a human gene of 6.5 kb to contain only a small open reading frame of 522 nucleotides. Unlike other members of the TNF family, VEGI is specifically expressed in endothelial cells. Northern analyses of total RNA preparations from 23 cell lines and primary cell cultures showed that VEGI mRNA was detected only in HUVEC and human venous endothelial (VE) cells (Fig. 2*B*). The mRNA of VEGI was not detected in a later passage of the venous endothelial (VE-2) and human artery endothelial cells. Analysis of a panel of primary endothelial cells derived from different organ origins showed that VEGI is expressed in subset of endothelial cells such as HUVEC and HMMVEC but not in HPAEC, HIAEC, or HCAEC (data not shown). These results indicate that only a subset of endothelial cells express the gene. The observation that VEGI is highly expressed in some vascularized tissues such as kidney but not in other vascularized tissues, such as heart, could be explained by the fact that VEGI is only detected in a subset of endothelial cells.

Potent *in vivo* anti-tumor activities of the soluble VEGI via gene therapy

The amino acid sequence of VEGI homologous to the TNF family prompted us to examine whether the novel gene has potential anti-cancer activity. In addition to the full-length VEGI, a secreted form of VEGI (VEGI/IL-6) was constructed by replacing the amino-terminal hydrophobic segment of VEGI (residues 1–22) with the IL-6 signal peptide and was cloned into the eukaryotic expression vector pcDNA3. The murine colon carcinoma MC-38 cells were transfected with expression vectors expressing

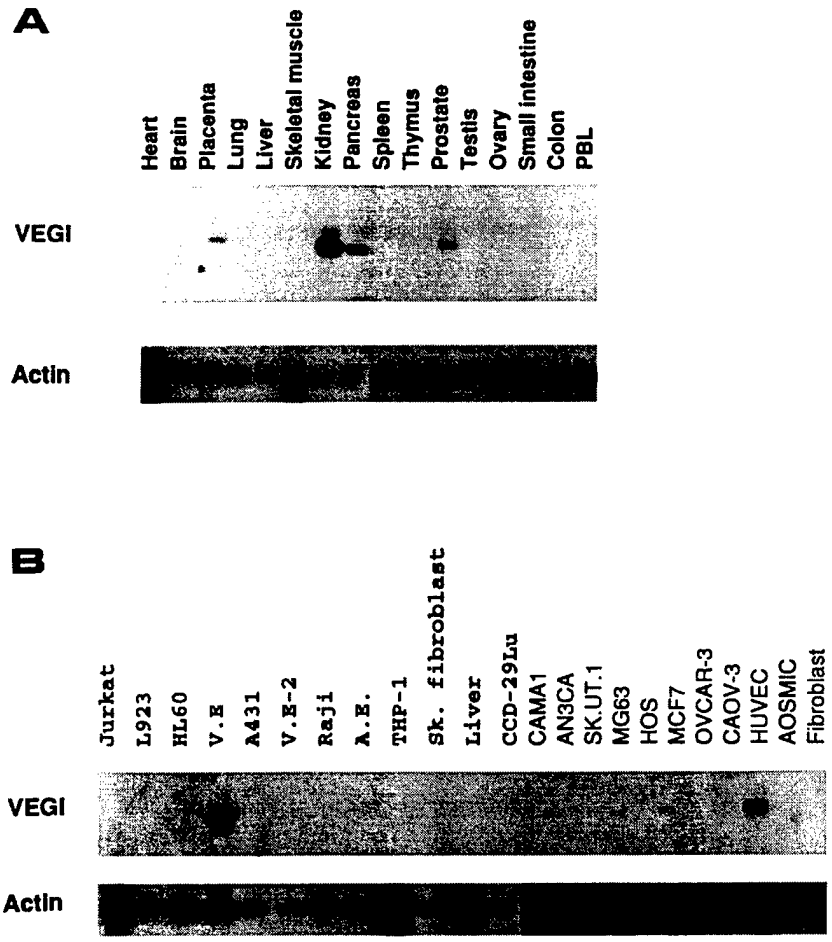

Figure 2. Northern blot analyses of VEGI transcript. *A*) VEGI expression in various types of the human tissues: the~6.5 kb VEGI and actin transcripts are indicated. *B*) VEGI expression in various types of the human cells: Jurkat, T cell leukemia cell; L923, embryonic kidney cell; HL60, promyelocytic leukemia; V.E, venous endothelial cell (early passage); A431, epidermoid carcinoma; V.E-2, venous endothelial cell (late passage); Raji, Burkitt's lymphoma; A.E., artery endothelial cell; THP-1, monocytic leukemia; CCD-29Lu, lung emphysema; CAMA1, breast cancer; AN3CA, uterine cancer; SK.UT.1, uterine cancer; MG63, osteoblastoma; HOS, osteoblastoma; MCF7, breast cancer; OVCAR-3, ovarian cancer; CAOV-3, ovarian cancer; HUVEC, human umbilical vein endothelial cell; AOSMIC, smooth muscle.

either full-length (VEGI) or the secreted form (VEGI/IL-6) of VEGI. Several stable cell lines expressing either the full-length VEGI or IL-6/VEGI were selected. Expression of VEGI on these tumor cells was confirmed by Northern blot analysis. VEGI transfection did not alter the *in vitro* proliferation rates of these tumor clones. These tumor cells were then subcutaneously injected into syngeneic C57BL/6 mice to test their abilities to form tumors. The two MC-38 stable cell lines expressing the full-length VEGI gene (VEGI-C1 and VEGI-C2) grew normally and caused rapid growth of tumors at a rate similar to that of wild-type MC-38 and cells transfected with the vector alone, MC-38/vector. On the other hand, all three cell lines expressing the secreted form of VEGI, including VEGI/IL-6-C1, VEGI/IL-6-C2, and VEGI/IL-6-C3, showed potent inhibition of the growth of MC-38 colon cancers in syngeneic C57BL/6 mice (**Fig. 3**). Thus, the replacement of the amino-terminal VEGI sequence with the IL-6 signal peptide, which make the VEGI protein soluble, led to suppression of tumor growth *in vivo* under the experimental setting. Our data indicated that the deletion of amino-terminal cytoplasmic and transmembrane domains of VEGI (residues 1–23) from the full-length protein appear to be critical for the inhibition of tumor growth *in vivo*.

The soluble VEGI reduces the microvessel density in tumors

Immunohistochemical analyses were performed to investigate the potential mechanisms of action for VEGI-mediated tumor rejection. As shown in **Fig. 4**, immunohistochemical staining with an antibody against vWF, a specific marker for the endothelial cells, revealed a significant reduction of the intratumoral microvessel density in the soluble VEGI/IL-6-

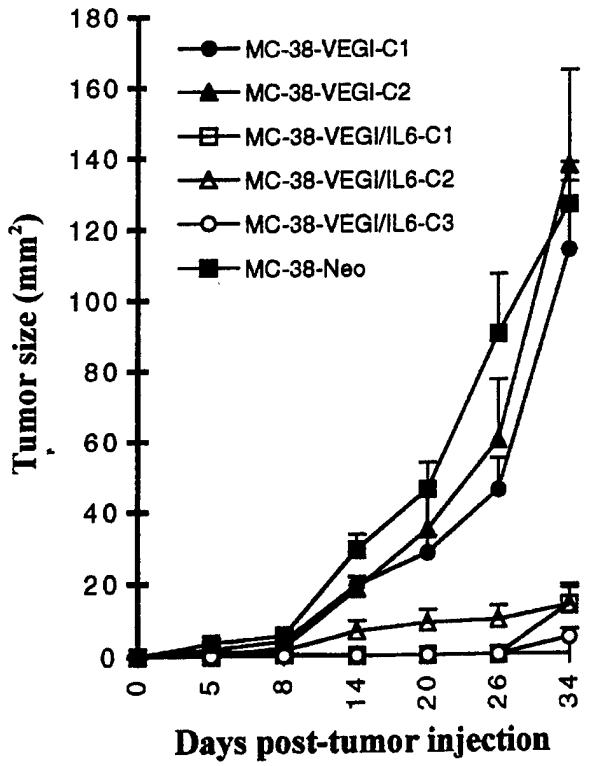

Figure 3. Effect of local production of the soluble VEGI via gene transfer on the growth of MC-38 murine colon carcinomas in syngeneic C57BL/6 mice. Female C57BL/6 mice were intradermally injected with 2×10^5 MC-38 cells transfected with either the full-length membrane-bound form (VEGI) or the soluble form (VEGI/IL-6) of VEGI or the neo vector control cells. Tumors were measured twice weekly in a coded, blinded fashion. Each point represents the mean±SD of nine mice in each group. The experiments were repeated with similar results.

expressing tumors (Fig. 4*f*), as compared with the neo control tumors (Fig. 4*b*) and tumors expressing the membrane-bound VEGI (Fig. 4*j*). Conversely, tumors with neither soluble nor membrane-bound VEGI recruit neutrophils and macrophages into the transfected tumors, as compared with the neo control tumors (Fig. 4*c, d, g, h, k, l*). Consistently, there was a significant decrease of microvessels in VEGI/IL-6-expressing tumors: the average numbers of vWF + endothelial cells (mean ±SD) per mm^2 tumor size in neo control, VEGI, and VEGI/IL-6-transduced MC-38 tumors were 241 ±34, 356 ±49, and 117 ±10, respectively ($P<0.03$), based on the immunohistological staining using the vWF mAb. In contrast, the mean values ±SD of Gr-1-positive neutrophils per mm^2 tumor size in neo control, VEGI, and VEGI/IL-6-transduced MC-38 tumors were 182 ±88, 160 ±104, and 102 ±81, respectively ($P>0.05$). These data suggest that the soluble form of VEGI, but not the membrane-bound VEGI, produced locally through transfection into tumor cells inhibits tumor-induced neovascularization *in vivo*. On the other hand, injection of VEGI-expressing tumor cells did not cause gross abnormalities in the syngeneic C57BL/6 mice, such as weight loss during the experiment period. Our data suggest that locally producing the soluble VEGI via gene transfer might exert a potent anti-tumor effect without inducing systemic toxicity.

VEGI inhibits the *in vitro* proliferation of the endothelial cells

To further investigate whether suppression of the *in vivo* tumor growth by the soluble VEGI was due to an anti-angiogenic effect or direct anti-tumor activity, the conditioned media were collected from cells expressing membrane-bound VEGI or the soluble VEGI/IL-6 and tested for their ability to inhibit the proliferation of MC-38 tumor cells or ABAE bovine aortic endothelial cells *in vitro*. As shown in **Fig. 5**, proliferation of ABAE endothelial cells stimulated by 1 ng/ml of bFGF was significantly inhibited by the conditioned media derived from the VEGI/IL-6-expressing cells. In contrast, no inhibitory activities on ABAE *in vitro* growth were observed in the conditioned media from the vector control or the membrane-bound VEGI-expressing cells. The data are consistent with the immunohistological studies demonstrating that the soluble VEGI/IL-6 inhibited intratumoral microvessel formation *in vivo*. However, overexpression of VEGI or VEGI/IL-6 in MC-38 tumor lines did not alter tumor cell growth rates *in vitro*. Thus, the soluble VEGI-mediated tumor rejection via gene transfer probably is at least in part due to its anti-angiogenic activity in inhibiting the endothelial cell proliferation.

DISCUSSION

Here we report the identification of a novel cytokine of the TNF family, VEGI. It is expressed specifically in endothelial cells, functions as an inhibitor of angiogenesis, and causes complete tumor inhibition in the syngeneic C57BL/6 mice via gene therapy.

The recent identification of the genes encoding TNF-like ligands and receptors has led to a better understanding of the roles and mechanisms of action of these molecules (1–5). To identify endothelial cell expression-specific gene(s), we have constructed 8 cDNA libraries using RNA derived from various endothelial cells, and generated approximately 30,000 ESTs. The ESTs were compared with ESTs in the human genome databases derived from a wide variety of types of cells. The ESTs unique to endothelial cells were further analyzed for sequence homology to the TNF family. VEGI was identified from an HUVEC cDNA library by EST database substraction. Like other TNF-related ligands, VEGI is a type-II transmembrane protein with the carboxyl terminus on the

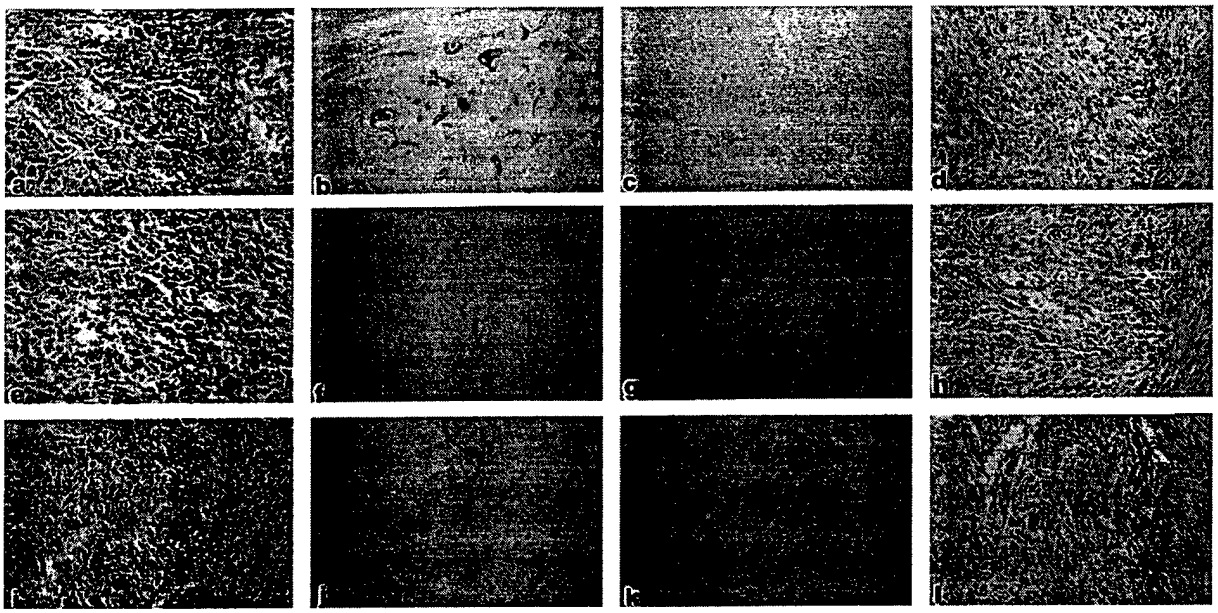

Figure 4. Histological evaluation of MC-38-neo (*a–d*), MC-38-VEGI/IL-6 (*e–h*), and MC-38-VEGI (*I–l*) murine colon cancers. The tumor sections were stained with either H & E (*a, e, I*), a rabbit anti-human von Willebrand factor (vWF) mAb that specifically recognizes the endothelial cells (*b, f, j*), a rat anti-mouse mAb Gr-1, which specifically recognizes neutrophils visualized by brown staining (*c, g, k*), or MMAC, a rabbit anti-mouse mAb that specifically recognizes the monocytes and macrophages *d, h, l*). Original magnification, ×40.

exterior cell surface, a single transmembrane domain, and a short cytoplasmic tail. It is a novel protein that exhibits 20–30% sequence homology to human TNFα, TNFβ, and the Fas ligand.

It is well documented that members of the TNF family display a wide variety of biological activities (1–5). Despite the functional redundancy of this family, specificity may be accomplished through restricted spatial and temporal expression of the ligands and their receptors to specific cell types. In this study, we described the identification of VEGI, a novel ligand of the TNF family, derived from the HUVEC endothelial cell cDNA library. Remarkably, VEGI expression is restricted to the endothelial cells according to Northern blot analysis and EST database search, which suggests that VEGI may play a role in the function of the normal vasculature.

TNFα is a potent cytokine that evokes multiple biological responses: it alters the proliferation, differentiation, and metabolism of a wide variety of cells (2) and activates the immune responses by activated macrophages, cytotoxic T cells, natural killer cells, and neutrophils (1, 14). The anti-tumor effects of TNFα have been well demonstrated by systemic administrations of the protein as well as by local production via gene transfer (14–16) . The homology of VEGI to the TNF family prompted us to examine whether the novel gene has potential anti-cancer activity. We approached this by using gene transfer in a syngeneic tumor model, since gene therapy has been valuable in assessing the anti-tumor effects of various cytokines (11, 17–19). As shown in Fig. 3, transgenic overexpression of the soluble VEGI completely suppressed the growth of MC-38 colon cancers in syngeneic C57BL/6 mice. However, no significant inhibitory effect on *in vitro* cell proliferation was observed in murine colon cancer MC-38 cells stably transfected with VEGI, suggesting that the expression of VEGI did not directly cause cytotoxicity in these tumor cells *in vitro*.

A number of possible mechanisms may account for the anti-tumor effects of VEGI observed in the syngeneic tumor model. The endothelial cell-specific expression of VEGI suggests of a possible mechanism by which the anti-tumor effect observed is mediated through the control of tumor angiogenesis. This working hypothesis of VEGI was further supported by our observation that immunohistologic staining with the endothelial-specific marker vWF reveals a significant reduction in the intra-tumoral microvessel density in VEGI/IL-6-transduced tumors. Consistently, the conditioned media obtained from cells expressing soluble but not the membrane-bound-VEGI specifically inhibit *in vitro* proliferation of ABAE endothelial cells, but not proliferation of MC-38 tumor cells. Thus, local production of the soluble VEGI via gene therapy shifts the net balance between angiogenic stimulators and inhibitors, thus inhibiting tumor growth *in vivo*. It remains to be seen whether other mechanisms, such as activation of tumor-specific or non-specific B or T lymphocytes or induction of cyto-

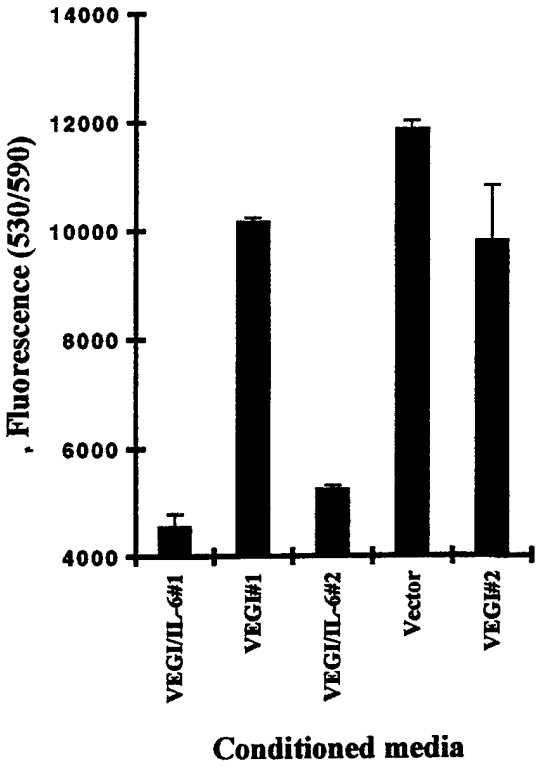

Figure 5. Effect of the conditioned media derived from the VEGI/IL-6-expressing cells on proliferation of the ABAE endothelial cells. ABAE cells were harvested and seeded in wells of a 96-well plate at 3000 cells/well in the appropriate growth medium containing 10% fetal bovine serum and 1 ng/ml of basic FGF. The conditioned media from various VEGI-expressing cells were then added into each well at a final 1:10 dilution. The cells were incubated in a final volume of 200μl for a 4–5 days. Alamar blue was added to each well to a final concentration of 10%. The cells are incubated for 4 h. Cell viability was measured by reading in a CytoFluor fluorescence reader with excitation at 530 nm and emission at 590 nm. All assays were done in triplicate.

kines, would also be involved in the soluble VEGI-mediated tumor rejection.

We have recently demonstrated that local production of LIGHT, a newly discovered TNF-like cytokine, caused marked infiltration of neutrophils into tumors (11). Our results are consistent with those from other laboratories in which CD95L-mediated neutrophil recruitment was responsible for the primary tumor rejection (17). However, such a phenomenon was not observed for VEGI-transfected tumors under our experimental setting. Whether the VEGI protein possesses similar activities under other conditions remains to be investigated. Regardless of the exact mechanisms of VEGI action, our results showed that a soluble version of VEGI, which correspond to the putative extracellular domain consisting of residue 23–174, is capable of inhibiting the growth of endothelial cells *in vitro*, and *in vivo*. Thus, VEGI may function as an angiogenesis inhibitor.

Angiogenesis is required for a variety of physiological processes such as organogenesis during fetal development, wound healing, and organ regeneration. Abnormal neovascularization leads to progression of many diseases such as cancers and diabetic retinopathy. Recent studies have also demonstrated that tumor growth and metastasis are angiogenesis dependent (20–22), and result from up-regulation of angiogenic stimulators such as VEGF or bFGF and/or down-regulation of angiogenesis inhibitors like endostain (20).

The *in vivo* anti-tumor effects of VEGI-gene therapy may be mediated by potentiating both anti-angiogenic and other nonspecific immune responses. Since the anti-tumor activity of VEGI was demonstrated by local production of the protein via gene transfer, additional studies including the administration of VEGI protein *in vivo* (which is not currently available) would be necessary to prove this conclusion. The generation of recombinant VEGI protein, development of antibodies against VEGI, and further identification of its natural forms as well as its receptor(s) should provide tools to understand the biological functions of VEGI.

In summary, we have identified and functionally characterized a novel member of the TNF superfamily. Our major findings are: *1*) VEGI is specifically expressed by the endothelial cells as demonstrated by the Northern blot analyses and supported by its unique presence in the expression tags isolated only from the endothelial cells; *2*) Local production via *in vivo* gene transfer of the secreted form, but not the membrane-bound form of VEGI, inhibits the growth of colon carcinomas in C57BL/6 mice; *3*) Histological examination of the tumors showed marked reduction of vascularization in MC-38 tumors that express soluble but not membrane-bound VEGI or when transfected with only the control vector. On the other hand, VEGI expression does not attract the neutrophils and macrophages to infiltrate into the tumor mass. *4*) The conditioned media from cells expressing soluble VEGI drastically inhibit *in vitro* proliferation of the ABAE cells. These results indicate that VEGI is a novel angiogenesis inhibitor of the TNF family and functions at least in part by directly inhibiting the proliferation of endothelial cells. Thus, this novel gene will be a valuable tool for future study of the molecular mechanism of vascular biology and will also serve as a potential target in the development of angiogenesis-based cancer therapy. FJ

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INHIBITION OF ANGIOGENESIS AND BREAST CANCER XENOGRAFT TUMOR GROWTH BY VEGI, A NOVEL CYTOKINE OF THE TNF SUPERFAMILY

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Recently, we reported a novel protein of the tumor necrosis factor (TNF) superfamily, named vascular endothelial cell growth inhibitor (VEGI), which is expressed predominantly in endothelial cells. When a secreted form of this new protein was overexpressed in mouse colon cancer cells, the growth of tumors formed by these cells in black mice was inhibited. We now report that recombinant VEGI inhibits the proliferation of endothelial cells but not that of other types of cells examined. The protein also inhibits formation of capillary-like structures by endothelial cells in collagen gels, and the growth of capillaries into collagen gels placed on the chick chorioallantoic membrane. The anticancer potential of VEGI was examined in a breast cancer xenograft tumor model in which the cancer cells were co-injected with Chinese hamster ovary cells overexpressing a secreted form of the protein. The co-injection resulted in potent inhibition of xenograft tumor growth. Our findings are consistent with the view that VEGI is an endothelial cell-specific negative regulator of angiogenesis. ***Int. J. Cancer*** **82:131–136, 1999.**
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The endothelium plays an essential role in the maintenance of vascular homeostasis and permeability. Endothelial cells are actively involved in inflammation, cell adhesion, coagulation, thrombosis, fibrinolysis and angiogenesis. During angiogenesis, endothelial cells proliferate, invade into stroma and migrate toward the source of an angiogenic stimulus such as cancer cells. They then interact with perivascular cells and stromal cells to eventually form capillaries (Folkman, 1995). Although the mechanism of angiogenesis regulation is not fully understood, it is becoming clear that the initiation or termination of the process is controlled by a balance between positive and negative regulators of angiogenesis. A number of angiogenic factors have been described including several members of the fibroblast growth factor family such as FGF-1 (Gimenez-Gallego *et al.*, 1985), FGF-2 (Schweigerer *et al.*, 1987), those of the vascular endothelial cell growth factor family (VEGF) (Leung *et al.*, 1989), and the angiopoietins (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997). The receptors of these growth factors and cytokines have been identified (Burrus and Olwin, 1989; de Vries, 1992; Sato *et al.*, 1995; Terman *et al.*, 1992; Wennstrom *et al.*, 1991). Several inhibitors of angiogenesis have also been reported, including thrombospondin (Good *et al.*, 1990), angiostatin (O'Reilly *et al.*, 1994), endostatin (O'Reilly *et al.*, 1997) and platelet factor-4 (Maione *et al.*, 1990).

Physiological angiogenesis as is seen *in utero*, in wound healing or in the female reproductive tract is coordinated, being activated promptly when required and curtailed rapidly when further angiogenesis is no longer appropriate in that physiological setting. By contrast, pathological angiogenesis as seen in tumors, rheumatoid arthritis or diabetic retinopathy, once initiated is prolonged and ongoing with no termination despite the formation of new vessels. The failure to curtail pathological angiogenesis implies that a negative regulatory mechanism, which is functioning in physiological angiogenesis, is missing or suppressed in pathological angiogenesis. As both autocrine and paracrine negative feedback systems are common mechanisms by which proliferative physiological processes are curtailed, it is plausible that endothelial cells, which are a main component of the vasculature, may produce factors to suppress angiogenesis under physiological conditions. No such endothelial cell-produced negative regulator of angiogenesis has been described previously.

We have reported the identification of a novel cytokine, vascular endothelial cell growth inhibitor (VEGI) (Zhai *et al.*, 1999). The *VEGI* gene encodes a protein of 174 amino acids that exhibits a 20–30% overall sequence homology to the tumor necrosis factor superfamily. Among a wide variety of human cells examined, VEGI mRNA was found by Northern blotting analysis to be expressed only in endothelial cells. Total RNA obtained from many adult human organs and tissues also contains VEGI mRNA, suggesting its expression by endothelial cells of the quiescent vasculature. The sequence homology of VEGI to the tumor necrosis factor (TNF) family members prompted us to examine its function in a cancer model. The presence of a highly hydrophobic segment (residues 16–25) near the *N*-terminus of the protein suggests that VEGI is probably a type II membrane protein with a brief intracellular *N*-terminal segment and most of the protein (residues 26–174) being an extracellular domain, like most TNF family members (Aggarwal and Natarajan, 1996). Because many TNF family members are cleaved from the membrane to function as soluble proteins to affect distant target cells with appropriate receptors (Black *et al.*, 1997; Kayagaki *et al.*, 1995), we assumed a similar mechanism for VEGI. A secreted form of VEGI was, therefore, overexpressed in murine colon cancer cells (MC-38). The transfected cancer cells had greatly decreased tumorigenicity when implanted in C57/BL mice. Inhibition of tumor angiogenesis was evident from much decreased microvessel density. The conditioned media of the transfected cancer cells were found to be able to inhibit endothelial cell proliferation.

We have now made recombinant protein consisting of the putative extracellular domain of VEGI in *Escherichia coli* and examined its activity in a variety of cellular and animal models. The protein is able to inhibit the growth of endothelial cells but not that of other types of cells examined. The protein also inhibits formation of capillary-like structures by endothelial cells in collagen gels, and the growth of capillaries into collagen gels placed on the chick chorioallantoic membrane (CAM). We have overexpressed a secreted form of VEGI in Chinese hamster ovary (CHO) cells and co-injected the CHO cells with human breast cancer cells in nude mice so that VEGI protein would be made available at the tumor site. The co-injection resulted in marked inhibition of the growth of the breast cancer xenograft tumors.

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These findings suggest that VEGI is a negative regulator of angiogenesis produced predominantly by endothelial cells.

MATERIAL AND METHODS

Cell lines

NIH 3T3 cells, CHO cells, human breast cancer cell lines MDA-MB-231 and MDA-MB-435 were purchased from the American Type Culture Collections (ATCC, Rockville, MD). Human umbilical cord vein endothelial cells (HUVE) and human vascular smooth muscle cells (HVSM) were purchased from Clonetics (San Diego, CA). Adult bovine aortic endothelial cells (ABAE) were a gift from Dr. P. Bohlen (ImClone, New York).

VEGI recombinant protein preparation

A truncated form of VEGI consisting of residues 29–174 was fused with thioredoxin, which enhances the solubility of VEGI in *E. coli*, and a poly-histidine tag in a pET32a plasmid (Novagen, Madison, WI). The protein was purified from the soluble fraction of the homogenate of *E. coli* by affinity chromatography using Ni-Sepharose (Bio-Rad, Hercules, CA) to apparent homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The thioredoxin segment can be removed by using thrombin; however, the presence of thioredoxin in the fusion protein had no effect on the activity of VEGI. The fusion protein was, therefore, used as an equivalent of VEGI in the assays reported in these studies.

Proliferation assay

Cells were seeded in triplicate at 8,000 cells/well in 24-well plates, in IMEM (GIBCO, Gaithersburg, MD), 10% fetal calf serum (FCS) and cultured at 37°C, 5% CO_2. ABAE and HUVE cell culture media contained additional 1 ng/ml and 6 ng/ml FGF-2, respectively. The media were changed once on day 3. The number of viable cells was determined on day 6 by using a Coulter (Hialeah, FL) counter.

In vitro *angiogenesis assay*

Quantitative assessment of capillary-like tube formation by endothelial cells cultured on collagen gels were carried out as described elsewhere (Li *et al.*, 1994; Montesano and Orci, 1985). Three-dimensional collagen gel plates (24-well) were prepared by addition to each well of 0.5 ml chilled solution of 0.7 mg/ml of rat tail type I collagen (Becton Dickinson Franklin Lakes, NJ) in IMEM containing 10% fetal bovine serum (FBS) and adjusted to neutral pH with $NaHCO_3$. After formation of the collagen gel (about 1–2 mm thickness), ABAE cells were seeded at 50,000 cells/well. The cultures were maintained in IMEM, 10% FCS, 1 ng/ml of FGF-2, 5% CO_2, 37°C for 72 hr. The media were then replaced with fresh media omitting FGF-2 and cultured for 48 hr. The media were then replaced with fresh media containing 20 ng/ml of FGF-2. The cultures were maintained at 37°C for 48 hr. The gels were then fixed with cold methanol (−20°C). The abundance of the capillary-like structures formed by ABAE cells was determined by computer-assisted image analysis, using an IM35 inverted Zeiss microscope with phase contrast and Hoffman optics. Image acquisition and analysis is carried out using the Optimas 5.2 software (Media Cybernetics, Silver Spring, MD). The relative intensity of the capillary-like structures were measured as the ratio of the total length of the tubular structures over the total areas measured (mm/mm^2).

Chicken embryo CAM angiogenesis assay

Collagen gel pellets (0.05 ml) were placed on CAM. Angiogenesis in the gel pellet was induced by FGF-2 (50 ng) or VEGF (100 ng) supplemented in the gels. Various amount of VEGI was also incorporated into the gels. The extent of angiogenesis was determined by evaluation of the fluorescence intensity of FTIC-dextran injected into the CAM circulation prior to the retrieval of the gels and retained in the gels, as described previously (Iruela-Arispe and Dvorak, 1997).

Cancer cell-CHO cell co-inoculation in nude mice

A fusion protein consisting the secretion signal of interleukin-6 (Hirano *et al.*, 1986) and residues 23–174 of VEGI was constructed by PCR (5′ primer: 5′-GCGGGATCCG CCACCATGAA CTCCTTCTCC ACAAGCGCCT TCGGTCCAGT TGCCTTCTCC CTGGGGCTGC TCCTGGTGTT GCCTGCTGCC TTCCCTGCCC CAGTTGTGAG AC-3′). The primer contains a BamH I restriction endonuclease site (underlined), the first 84 bases of interleukin-6 coding sequence and 18 bases of VEGI starting from Pro23; 3′ primer: 5′-CGCGGATCCG ATATTTGCTC TCCTCCTCA-3′, containing a BamH I restriction endonuclease site (underlined) and a stop codon and was inserted into an expression vector (pC1) that carries the dihydrofolate reductase gene. The plasmid was transfected into CHO cells that are dihydrofolate reductase negative. The stable CHO cell clones were selected for their ability to resist methotrexate, a dihydrofolate reductase inhibitor. The transfectants were maintained in modified Eagle's medium (MEM)-α containing 10% dialyzed FCS. CHO cell clones were further selected based on the presence of VEGI mRNA determined by Northern blotting analysis and the ability of the conditioned media, upon concentration, to inhibit ABAE cell growth. A quantitative assessment of VEGI protein in the conditioned media was not possible because of the lack of suitable antibodies. To ensure adequate production of VEGI at the tumor sites, 5×10^6 vector-transfected or secreted VEGI-overexpressing CHO cells were mixed with 10^6 human breast cancer cells (MDA-MB-231 or MDA-MB-435), an amount usually used in our inoculation of breast cancer cell lines. The cell mixtures were then injected into the mammary fat pads of female nude mice. Tumor sizes (mm^2) were measured in a blinded manner twice a week following injection.

RESULTS

Specific inhibition of endothelial cell proliferation by recombinant VEGI

A truncated form of VEGI consisting of residues 29–174 was expressed in *E. coli*. Among various truncations tested, the one reported here is the most active recombinant protein thus far. The protein was found to preferentially inhibit the FGF-2-induced proliferation of ABAE and HUVE while having no effect on the growth of HVSM, human breast cancer cells (MDAMB231) or NIH 3T3 cells (Fig. 1). The half-maximum inhibitory concentrations (IC_{50}) for ABAE and HUVE cells were about 6 ng/ml and 60 ng/ml, respectively. The difference in the IC_{50} values between the 2 cell types of different species probably reflects different affinities of the recombinant VEGI toward the receptors on the cell surface. The protein also had no effect on the proliferation of human T cells or bone marrow stromal cells at 100 ng/ml. These results suggest that VEGI, which is predominantly expressed by endothelial cells, specifically inhibits proliferation of endothelial cells when compared with other cell types tested.

Inhibition of in vitro *angiogenesis*

The anti-angiogenic activity of the recombinant VEGI was examined with an *in vitro* angiogenesis model. In this model, ABAE cells growing on a 3-dimensional collagen gel form a monolayer on the surface when the cell culture reaches confluence (Fig. 2*b*). Upon stimulation of the confluent monolayer cells with an angiogenic factor such as FGF2, however, many cells invade into the gel and form capillary-like tubular structures in the gel (Fig. 2*c*). When recombinant VEGI was added to the cell cultures together with FGF-2, inhibition of the formation of capillary-like tubes by ABAE cells was observed (Fig. 2*d*). The relative intensities of the tubules were determined by using computer-assisted image analysis. The IC_{50} value for the inhibition was found

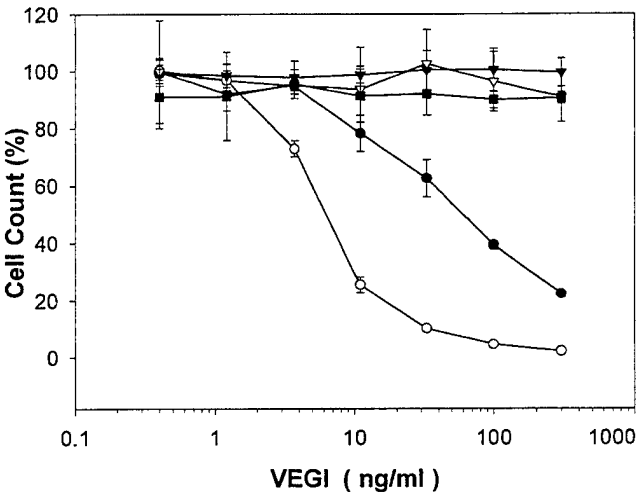

FIGURE 1 – Specific inhibition of endothelial cell proliferation by vascular endothelial cell growth inhibitor (VEGI). ●: human umbilical cord vein endothelial cells (HUVE); ○: adult bovine aortic endothelial cells (ABAE); ▼: human vascular smooth muscle cells (HVSM); ▽: human breast cancer cells (MDAMB231); ■: NIH3T3 mouse fibroblast cells. Cells were seeded in triplicate at 8,000 cells/well in 24-well plates. Culture conditions are described in Material and Methods. The media were changed once on day 3. The number of viable cells was determined on day 6 by using a Coulter counter. Mean values and standard deviations are presented as a function of VEGI concentrations.

to be approximately 30 ng/ml (Fig. 2*a*). Little cytotoxicity was observed on the confluent endothelial cells in the background monolayer (Fig. 2*d*). These results may have arisen from inhibition of proliferation and of certain differentiating functions involved in tubule formation.

Inhibition of chick CAM angiogenesis

The anti-angiogenic activity of VEGI was further determined by using a modified CAM assay. The method is based on the vertical growth of new capillary vessels into a collagen gel pellet placed on the CAM. The collagen gel was supplemented with an angiogenic factor such as FGF-2 (50 ng per gel or 1.0 µg/ml) or VEGF (100 ng per gel or 2.0 µg/ml), with or without VEGI. The high concentrations of the angiogenic factors were necessary to maintain a gradient in order to induce significant growth of capillaries into the gels. Because the size of the gel pellets (50 µl) was negligible, the angiogenic factors or inhibitors supplemented in the gels had no effect on the development of the CAM vasculature or that of the chick embryos. The extent of angiogenesis in the gel was assessed by using fluorescein isothiocyanate (FITC)-dextran, a fluorescent dye, injected into the circulation of the embryo. Simultaneously supplementing VEGI in the gels gave rise to a marked inhibition of the capillary growth in a dose-dependent manner. The concentration for VEGI to achieve half-maximum inhibition on FGF-2-induced angiogenesis was about 50 ng per gel (Fig. 3*a*). The half-maximum inhibitory concentration for VEGI to inhibit VEGF-induced angiogenesis was about 100 ng per gel (Fig. 3*b*). These IC_{50} values reflect a nearly one-to-one molar ratios between the stimulator and the inhibitor, because the m.w. of FGF-2, VEGF and VEGI are similar. These results indicate that the recombinant VEGI

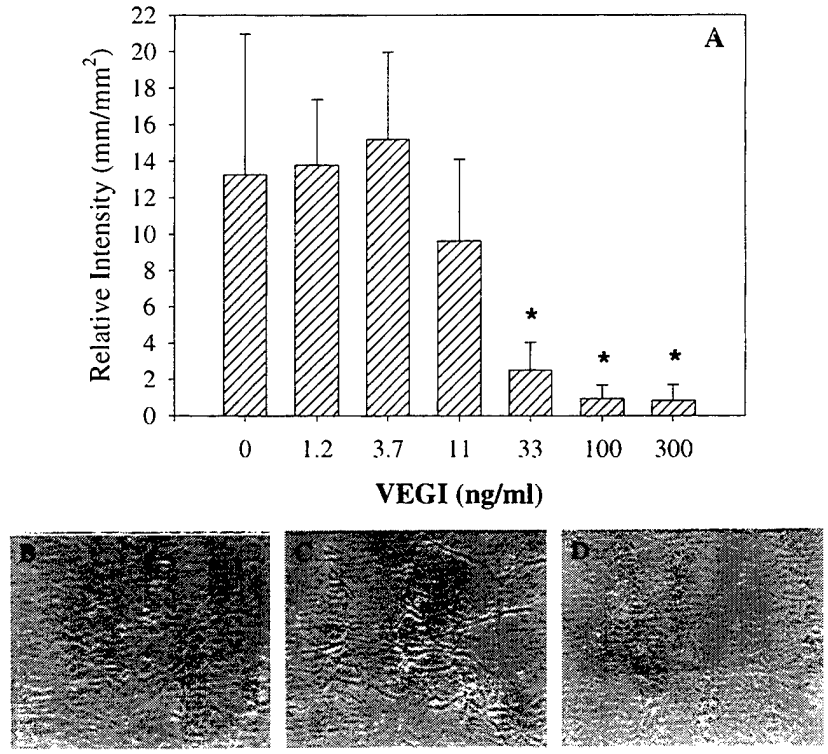

FIGURE 2 – Inhibition of formation of capillary-like tubules by adult bovine aortic endothelial cells (ABAE) cells in collagen gels. (*a*) Ability of vascular endothelial cell growth inhibitor (VEGI) to inhibit the formation of capillary-like tubes by ABAE cells is shown. The experiments were carried out in triplicate. At least 9 image areas were analyzed for each VEGI concentration. Mean values and standard deviations are presented as a function of VEGI concentration. Asterisks indicate $p < 0.05$ (ANOVA, Tukey test) by comparing to the tube intensity in the absence of VEGI. (*b*) ABAE cell monolayer on collagen gel. (*c*) Capillary-like tubes formed by ABAE cells when induced by fibroblast growth factor (FGF)-2 (50 ng/ml). Notice that the tube-like structures are on a different focal plane than the cell monolayer in the background. (*d*) Few tube-like structures are formed when VEGI (100 ng/ml) is present in the culture media. Notice the normal appearance of ABAE cells in the monolayer.

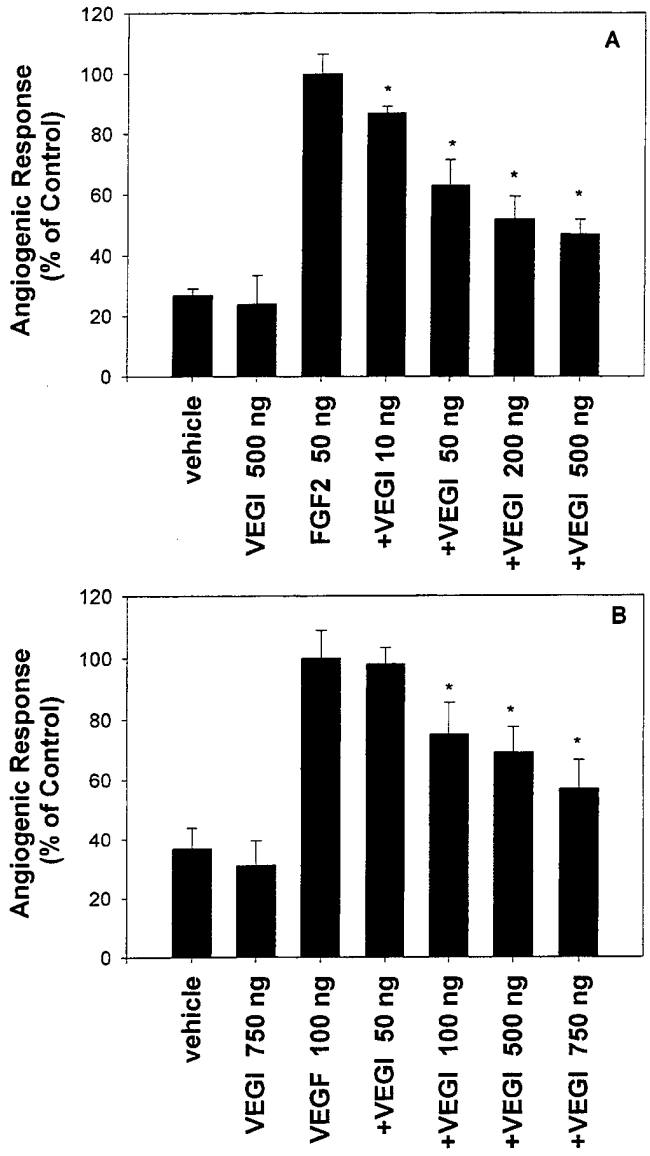

FIGURE 3 – Inhibition of chick embryonic chorioallantoic membrane (CAM) angiogenesis. (*a*) Inhibition of fibroblast growth factor (FGF)-2-induced angiogenesis in collagen gels placed on CAM. Angiogenesis in the gel pellet (0.05 ml) was induced by FGF-2 (50 ng) supplemented in the gels. Various amount of vascular endothelial cell growth inhibitor (VEGI), as indicated, was also incorporated into the gels. The extent of angiogenesis was determined by evaluation of the fluorescence intensity of FTIC-dextran injected into the CAM circulation before retrieval of the gels and retained in the gels. Error bars represent the standard deviation of quadruplicate experiments for each VEGI concentration. Asterisks indicate $p \leq 0.05$ (ANOVA, Tukey test) by comparing extent of FGF-2-induced angiogenesis. (*b*) Inhibition of vascular endothelial cell growth factor family (VEGF)-induced angiogenesis in the CAM under experimental conditions identical to described above, except that VEGF (100 ng) was used to induce angiogenesis.

was able to inhibit capillary growth regardless the cause of the angiogenic process.

Inhibition of human breast cancer xenograft tumor growth by CHO cells overexpressing VEGI

Because a negative regulator of angiogenesis would be expected to exert a inhibitory effect on tumor growth, the potential anticancer activity of VEGI was investigated, using breast cancer cells that are highly tumorigenic when implanted into the mammary fat pads of female athymic nude mice. Because VEGI would normally be found on endothelial cell membrane, perhaps in close juxtaposition to its receptor on the same cells, an *in vivo* experiment to test its anti-angiogenic properties might necessitate transduction of host endothelial cells with viral expression vectors. To avoid this experimentally difficult problem, and to deliver active VEGI to a tumor and surrounding stroma, a secreted form of VEGI was constructed by replacing the *N*-terminal segment containing the putative transmembrane of VEGI with a secretion signal peptide derived from human interleukin-6 (Hirano *et al.*, 1986). Because the secretion signal peptide is cleaved in the secretion vesicles, this strategy should give rise to the production of the same VEGI species used in the *in vitro* studies. The construct was transfected into CHO cells (clone 1.2). Expression of the corresponding mRNA was confirmed by Northern analysis. Secretion of the modified VEGI by the transfected cells was confirmed by the ability of the CHO cell-conditioned medium to inhibit ABAE cell growth when added to ABAE cultures, whereas no inhibitory activity was found in the conditioned media of full-length VEGI transfected cells (data not shown). This finding is consistent with our earlier finding that tumor cells transfected with full-length VEGI retained unaffected tumorigenicity (Zhai *et al.*, 1998), again applying that VEGI needs to be delivered to endothelial cells. The transfected CHO cells were mixed with human breast cancer cells (MDAMB231 or MDAMB435), and the cell mixtures were injected into the mammary fat pads of nude mice. A marked inhibition of the growth of the xenograft tumors formed by either the MDA-MB-231 (Fig. 4*a*) or the MDAMB435 cells (Fig. 4*b*) was observed. Vector-transfected CHO cells had no effect on tumor growth in either case. The experiments were repeated and similar results obtained. No tumor growth was observed when the transfected CHO cells were implanted alone (data not shown).

The experiment was repeated with another stable VEGI-overexpressing CHO cell line (clone 2.9). Interestingly, when MDA-MB-231 cells were co-injected with these CHO cells, there was an initial tumor growth in all animals; however, 5 of 6 animals showed marked decrease of the tumor sizes within 1–2 months (Fig. 4*c*). In sharp contrast, all animals had progressive tumor growth in the control group in which the cancer cells were co-injected with vector-transfected CHO cells (Fig. 4*d*). The experiments were repeated and similar results obtained. No tumor growth was observed when clone 2.9 cells were implanted alone (data not shown).

DISCUSSION

The activity of the recombinant VEGI was examined initially in a variety of biological assays, including the proliferation of different cell types. The only activity found to be associated with VEGI is the inhibition of endothelial cell proliferation. The potency of the inhibition was remarkable. Interestingly, no inhibitory effect was found on other types of cells examined under similar concentrations. This finding indicates that the target cells of VEGI are primarily endothelial cells. The ability of VEGI to suppress angiogenesis was illustrated with cellular and animal models. The formation of capillary-like tubules by endothelial cells in collagen gels is a process that exhibits many of the elemental endothelial cell activities during angiogenesis in addition to proliferation. The endothelial cells form a network of capillary-like tubular structures underneath a monolayer of confluent cells, which remains on the surface of the gel. The recombinant VEGI was able to prevent the tubule formation without apparent cytotoxic effect on the confluent cells. This result is in agreement with the fact that *VEGI* is expressed in many normal adult tissues, suggesting that this gene may be involved in suppressing the proliferation and differentiation of endothelial cells in a normally quiescent vasculature in adults. The results from the CAM model confirmed that VEGI can prevent neovascularization *in vivo*. In addition, inhibition of CAM angio-

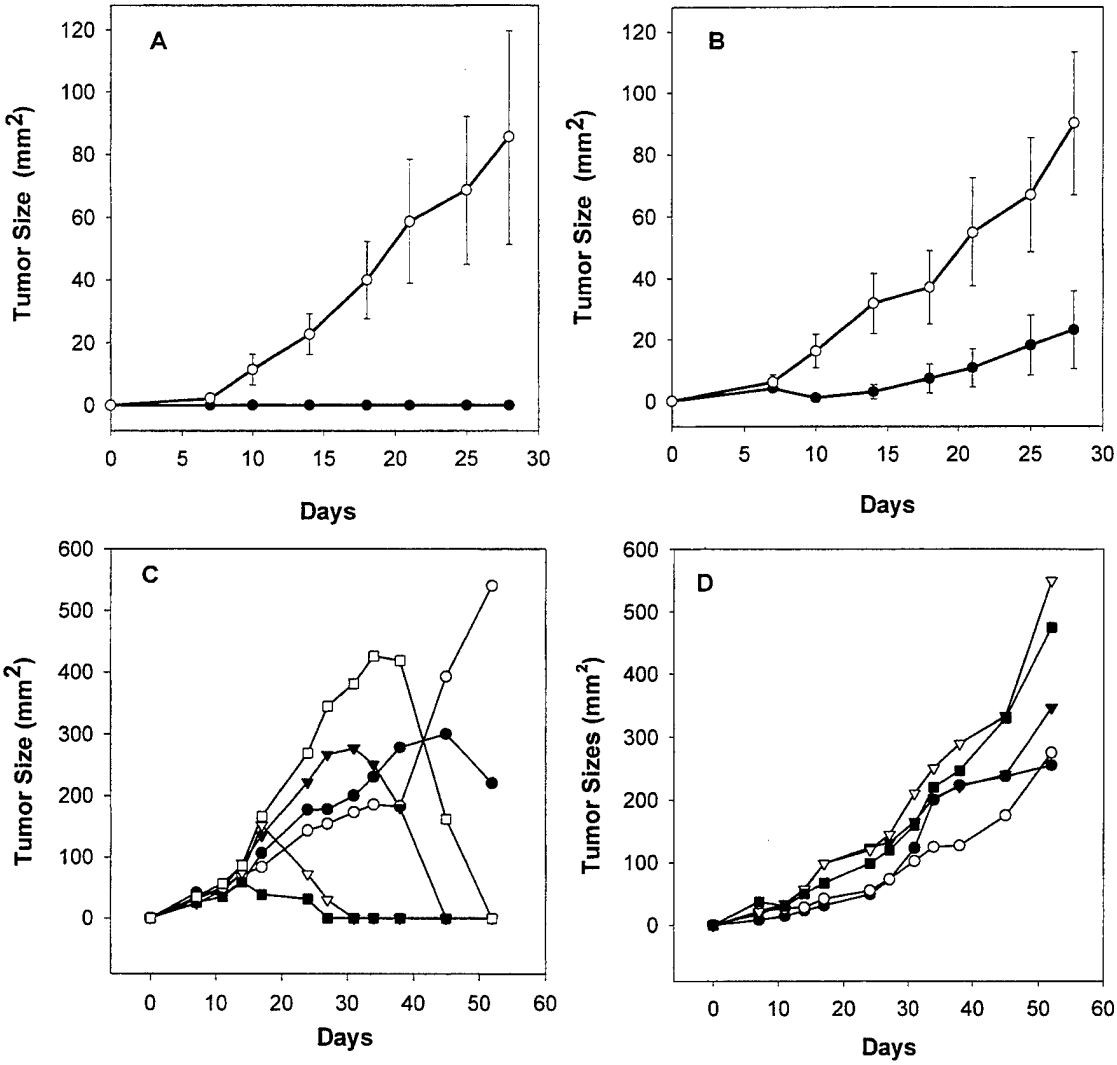

FIGURE 4 – Inhibition of the growth of 2 different human breast cancer xenograft tumors in nude mice by co-injected Chinese hamster ovary (CHO) cells overexpressing a secreted form of vascular endothelial cell growth inhibitor (VEGI). Vector-transfected or secreted VEGI-overexpressing CHO cells (5×10^6 (clone 1.2) were mixed with 10^6 cancer cells, then injected into the mammary fat pads of female nude mice (6 mice per group). Tumor sizes (mm^2) were monitored in a blinded manner. Mean values and standard deviations are presented as a function of days post-injection in (*a*) and (*b*). The experiments were repeated at least 2 times. Data shown were from one representative experiment. (*a*) Sizes of tumors formed by MDA-MB-231 cells co-injected with vector-transfected (○) or VEGI-producing CHO cells (●). (*b*) Sizes of tumors formed by MDA-MB-435 cells co-injected with vector-transfected (○) or VEGI-producing CHO cells (●). (*c*) Size of 6 individual tumors formed by MDA-MB-231 cells co-injected with VEGI-producing CHO cells (clone 2.9) in the experimental group. (*d*) Size of 5 individual tumors formed by MDA-MB-231 cells co-injected with vector-transfected CHO cells in the control group.

genesis by VEGI took place regardless what angiogenic factor induced the capillary growth. These results support the notion that VEGI is most likely not competing for the receptors of these very different growth factors, but exerting its activity by binding to a specific cell surface receptor to initiate a unique signaling pathway that would lead to the termination of angiogenesis.

The ideal method to demonstrate the potential anticancer effect of VEGI is a direct assessment of the recombinant protein in animal cancer models, but as yet we have been unable to prepare a sufficient amount of the protein to carry out this study. The CHO cell-cancer cell co-inoculation model was used as an alternative means of protein delivery. The results demonstrated that the release of VEGI into the vicinity of tumor cells leads to the inhibition of tumor growth. The lack of activity of full-length VEGI transfection of CHO cells or MC-38 mouse colon cancer cells also supports this conclusion. The striking anti-tumor effect of VEGI was likely to result from the ability of VEGI to suppress neovascularization, because recombinant VEGI had no inhibitory activity on the growth of the cancer cells *in vitro*, and secretion of VEGI by the transfected CHO cells had no inhibitory effect on their own growth. Furthermore, as shown by the collapse of the newly established xenograft tumors formed by the mixture of the cancer cells and the VEGI-overexpressing CHO cells, the action of VEGI probably led to eradication of tumor vasculature which is characterized by constant proliferation of endothelial cells. Similar eradication of newly established xenograft tumors was also observed when VEGI-overexpressing CHO cells (clone 1.2) were co-injected with prostate cancer PC3 cells. We demonstrated a dose-dependent tumor inhibition by altering the ratio of CHO:PC3 cells in the initial inoculum (data not shown). It is plausible that the amount of VEGI produced by co-injected CHO cells was initially insufficient to prevent the formation of the xenograft tumors; however, the CHO cells may grow together with the cancer cells in the tumors until a sufficient amount of VEGI was produced to effectively

terminate tumor neovascularization, which then led to the eradication of the tumors. Initially, the admixed tumor cells would provide the angiogenic stimulus for the growth of the CHO cells. It has been shown that CHO cells transfected with VEGF acquire the ability to proliferate dominantly in nude mice to form well-vascularized lesions, and the effect was attributed to the angiogenic activity of VEGF through paracrine mechanisms, because the CHO cells were not transformed (Ferrara *et al.*, 1993).

In conclusion, we presented evidence that VEGI, a novel cytokine predominantly expressed in endothelial cells, is a potent inhibitor of angiogenesis and tumor growth. Our findings are consistent with the view that VEGI may function as a negative regulator of angiogenesis involved in the maintenance of quiescence of normal vasculature or the termination of neovascularization. This is an example of an antiangiogenic factor produced by and acting on endothelial cells.

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